

土壌殺菌剤ハイメキサゾール,3-ヒドロキシ-5-メチルイソキサゾールのキュウリ蔓割病菌に対する殺菌作用機序

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Mode of Action of Soil Fungicide Hymexazol, 3-Hydroxy-5-Methylisoxazole, on *Fusarium oxysporum* f. *cucumerinum*

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Abstract

Fusarium oxysporum f. *cucumerinum* was sensitive to hymexazol (3-hydroxy-5-methylisoxazole, soil fungicide, Tachigaren®), whereas *Saccharomyces cerevisiae* was tolerant. Growth of *F. oxysporum* f. *cucumerinum* in the liquid culture medium was considerably inhibited by 300 µg/ml of hymexazol incorporated in the medium, while the growth of *S. cerevisiae* was not inhibited even at a concentration of 1,000 µg/ml. Differences in toxicity of hymexazol to these microorganisms primarily resulted from difference in uptake. No detectable metabolic conversion of hymexazol occurred in cultures of *S. cerevisiae*. Oxygen uptake by the intact cells of *F. oxysporum* f. *cucumerinum* was not greatly inhibited by concentrations of hymexazol that prevented growth of the fungus. Furthermore, the oxidation of succinate in mitochondrial preparations of the fungus was not significantly sensitive to the toxicant nor was respiration of intact cells. In addition, hymexazol at 500 µg/ml did not affect oxygen uptake by mitochondria derived from rat-liver. A concentration of hymexazol giving great inhibition of growth (500 µg/ml) did not cause detectable change in the permeability of the cells. Incorporation of phenylalanine into protein and uridine into ribonucleic acid (RNA) were inhibited considerably. Incorporation of thymidine into the deoxyribonucleic acid (DNA) fraction of the mycelial cells was inhibited more than phenylalanine and uridine into protein and RNA respectively. From these results, it appeared that hymexazol inhibits the fungal growth by interfering with the DNA synthesis as primary site of action.

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Introduction

Hymexazol (3-hydroxy-5-methylisoxazole, Tachigaren®) is a new soil fungicide, and has been used for control of soil-borne diseases, such as *Fusarium* wilt and damping-off of rice seedlings (*Fusarium* spp., *Pythium* spp., and *Rhizotonia* spp.), cucumber (*Fusarium oxysporum* Schlecht. ex Fr. f. *cucumerinum* Olen and *Pythium aphanidermatum* Edson), tomato [*Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyder & Hans.), and beets (*Pythium debaryanum* Hesse)¹⁸⁾.

The absorption and translocation pattern of hymexazol in plants has been studied by the authors¹⁰⁾. Its ability to enter and be translocated in plant tissue is a desirable property of this chemical. The metabolic fate of hymexazol has also been reported^{8, 9)}, and an interesting evidence for the possible conversion of hymexazol

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to compounds with hormonal qualities was observed^{5, 15)}. Disease control can be obtained by applying the chemical as a seed coat, or as drench pre-and post-planting treatment. Diseases incited by *Fusarium* spp. are particularly troublesome in plant cultivation, and their control is a primary concern.

The objectives of this investigation were to determine the mechanism of antifungal action of hymexazol. Results pertaining to the uptake and metabolism of the toxicant are to be published elsewhere⁶⁾. Portions of these studies were reported previously⁷⁾.

Materias and Methods

Chemical. Crystalline hymexazol, 3-hydroxy-5-methylisoxazole, which was synthesized by K. Tomita *et al.* in our Agricultural Chemicals Laboratories, was dissolved in sterile distilled water at an appropriate concentration and used throughout this study.

Test fungi. *F. oxysporum* Schlecht. ex Fr. *f. cucumerinum* Oven (F-16) used in the experiments was obtained from the collection of Sankyo Agricultural Chemicals Research Laboratories. The strain was maintained and subcultured in F-medium as previously described⁶⁾. After successive subculturing, young growing mycelial cells were obtained from 4-day cultures in Fernbach flasks, and used in all experiments. *Saccharomyces cerevisiae* Hansen (SANK 50361) was obtained from the collection of Sankyo Fermentation Research Laboratories and conditions for culturing of this strain have also been described previously⁶⁾.

Growth inhibition curve. Spore and mycelial suspension of the Fusarial fungus was incubated to 10 ml of the F-medium⁶⁾ in 50-ml L-flasks on a reciprocal shaker at 27 C. An aliquot 0.1 ml of hymexazol solution was added to the flasks at zero time. After growing for appropriate periods of time, the mycelia were harvested by filtration onto previously weighed filter papers and then transferred to an oven maintained at 100 C. The determinations of dry weight were made after 24 hr. *S. cerevisiae* was grown in the yeast medium⁶⁾ at 30 C on a reciprocal shaker. The spore suspension prepared was used for growth inhibition tests. The growth of *S. cerevisiae* was measured photometrically according to the procedure of Siegel and Sisler¹⁶⁾.

Respiration of intact cells of *F. oxysporum f. cucumerinum*. Respiration was measured polarographically in a semiclosed reaction vessel of an oxygen meter of Beckman Co. (O₂ Macroelectrode, Spinco Division of Beckman Inst., USA), originally designed by Hagiwara²⁾. The reaction mixture in the vessel contained 150 μ moles of phosphate buffer solution (pH 6.5) and the suspended cells equivalent to 1 to 2.5 mg (dry weight) in a total volume of 1.5 ml. The incubation was started by the addition of cell suspension and carried out at 27 C with stirring. The oxidation reaction was followed for several minutes on a recorder. Per cent of inhibition was calculated from the slope on the chart before and after the addition of hymexazol or other reference toxicants.

The fresh and active growing cells of Fusarial fungus prepared as indicated previously were used in all experiments. A part of the intact cells used was starved cells in order to increase their sensitivity to the toxicant. The starved cells were prepared by incubating intact cells for 48 hr at 27 C on a reciprocal shaker in phosphate buffer solution (pH 6.5) without the addition of other nutrients (see Fig. 2-A).

The effects of substrate on respiration of the intact cells, as well as mitochon-

dria, were tested with glucose, fructose, xylose, galactose, lactate, L-malate, succinate, ascorbate, citrate, fumarate, α -ketoglutarate, α -glycerophosphate and β -hydroxybutyrate. Since the effect of glucose was most clear, it was used as substrate for respiration by the intact cells, as seen in Fig. 2.

Preparation of *F. oxysporum* f. *cucumerinum* mitochondria. Young mycelial cells were ground in a mortar with two volumes of silica sand and then suspended in 500 mM sucrose, 10 mM sodium ethylenediaminetetraacetate (EDTA), 0.15% bovine serum albumin, 6 mM potassium citrate, and 10 mM Tris buffer adjusted to pH 6.5. The suspension was first centrifuged at $1,000 \times g$ for 10 min, and the pellet was discarded. The supernatant solution was then centrifuged at $10,000 \times g$ for 10 min, and the resultant pellet was resuspended in the medium mentioned above. The suspension was recentrifuged at $1,000 \times g$ for 10 min, and the supernatant suspension, which had a final concentration of 10 to 30 mg of protein per ml, was referred to as mitochondrial fraction. Experiments with the mitochondria were conducted on the day they were prepared since respiratory control index (RCI) of the fungal mitochondria had been found to decrease within several hours after their preparation even at 0 C¹⁴⁾. All operations were carried out between 0 to 4 C. Protein in the mitochondrial fraction was determined by the method of Lowry *et al.*¹¹⁾.

Oxidative phosphorylation by mitochondria of *F. oxysporum* f. *cucumerinum*. Oxygen uptake was measured in the oxygen meter at 27 C. A 1.5-ml amount of the basal medium (pH 6.5, adjusted with KOH), containing 125 mM sucrose, 10 mM KH₂PO₄, 10 mM MgCl₂, 1.5 mM EDTA, and 4.5 mg of bovine serum albumin, was first transferred to the vessel and incubation was started by addition of 20 to 100 μ l of mitochondrial suspension (1 to 2 mg of protein). Substrate (20 μ l), adenosine diphosphate (ADP; 10 μ l), and inhibitors (15 μ l) were also added to the incubation mixture. RCI was calculated from the rates of oxygen uptake before and after the addition of ADP. Effect of inhibitors was followed in the presence of both substrate and ADP, and per cent of inhibition was calculated as described above. In order to assay oxidative phosphorylation, about 5 μ Ci of orthophosphate, 0.5 mg of hexokinase (Sigma Chemical Co., type 4), and 150 μ M of glucose were added to the reaction mixture. At predetermined intervals 100 μ l samples of the incubation mixture were taken out for the assay of adenosine triphosphate (ATP), which was carried out by the method of Nielsen *et al.*¹³⁾ Free orthophosphate was determined by Takahashi's method¹⁷⁾.

Preparation of rat mitochondria. Two rat livers (50.48 g, fresh weight) obtained from male rat of strain of Wistar-Imamichi were washed twice in cold distilled water, homogenized by Potter-Elvehjem homogenizer and suspended in the preparation medium consisting of 0.25 mM sucrose and 3 mM EDTA [pH 7.2, adjusted with Tris (hydroxymethyl)-aminomethane]. The suspension was first centrifuged at $700 \times g$ for 10 min, and the pellet was discarded. The supernatant solution was then centrifuged at $5,000 \times g$ for 10 min and the resultant pellet was resuspended in the medium mentioned above. The suspension was centrifuged at $10,000 \times g$ for 10 min, three times. The pellet finally resuspended in 5 ml of the same medium is referred to as mitochondrial fraction. Experiments with rat mitochondria were conducted on the day they were prepared at 0 to 4 C.

Oxygen uptake in rat mitochondria. Oxygen uptake was measured in the same manner as described above in the basal medium containing 250 mM sucrose, 0.1 mM EDTA, 2 mM MgCl₂ MgCl₂·6H₂O 6H₂O, 20 mM KH₂PO₄ and 10 mM KCl (pH 7.2 adjusted with KOH). The basal medium (1.5 ml) was first transferred to the

vessel. Incubation was started by addition of 100 μ l of mitochondrial suspension (5 mg of protein). Substrate (20 μ l), ADP (10 μ l) and inhibitors (15 μ l) were also added to the incubation mixture. By the repeated addition of ADP and substrate, the ADP response by mitochondria was measured, and proved to be efficient. RCI was calculated as mentioned above. Effect of inhibitors was followed in the presence of substrate.

¹⁴C-glucose leakage. Young mycelial cells of *F. oxysporum* f. *cucumerinum* (100 mg, dry weight) were suspended in 100 ml of the phosphate buffer solution (pH 6.5) in a 500-ml Sakaguchi flask. The cell suspension was incubated for 2 hr at 27 C by shaking. ¹⁴C-glucose-(U) (specific activity; 3.9 mCi/mM, 12 μ M/g fresh weight cells) was added to the suspension at zero time. Feeding of labeled glucose to the cells was made for exactly 2 hr, and stopped by separation of the cells from the medium by filtration. The cells were thoroughly washed with the phosphate buffer more than ten times or until radioactivity was no longer detectable in the washing solution. Thereafter, a part of the cells (1/5) was used for measuring both radioactivity and dry weight. A certain amount of the cells that absorbed ¹⁴C-glucose was homogenized in a blender, extracted three times with methanol, each at 5 min, and suspended homogeneously in dioxane-toluene scintillation fluid supplemented with 3% Cab-O-Sil (Packard Instrument Co., USA), and measured for radioactivity. The rest of the cells (4/5) that had absorbed ¹⁴C-glucose were suspended in the fresh phosphate buffer solution (pH 6.5). The cell suspension prepared as 10 ml in a 50-ml L-flask was incubated at 27 C on a reciprocal shaker. An aliquot (0.1 ml) of hymexazol solution (final concentration; 50 and 500 μ g/ml) or p-s-butylphenol (200 μ g/ml, referential compound⁴⁾) was added to each of the flasks. Then, 0.5 ml aliquot of the cell suspension was taken out at hourly intervals, and centrifuged at 1,000 $\times g$ for 10 min. The supernatant solution was suspended in dioxane-toluene scintillation fluid, and the radioactivity derived from the leakage of membrane was determined. Further investigation on this respect was made with ³²P-o-phosphate (0.038 μ Ci) in the same experimental system as above.

Phenylalanine incorporation. Experiments below principally followed to the procedure of Hock and Sisler in the case of *Rhizoctonia solani*³⁾ with some modifications. Mycelial cells of *F. oxysporum* f. *cucumerinum* (10 g, fresh weight) were incubated in 100 ml of phosphate buffer solution (pH 6.5) in a 500-ml Sakaguchi flask containing 100, 300 or 1,000 μ g/ml hymexazol. After 3 hr incubation, 0.8 μ M 3-¹⁴C-L-phenylalanine with a specific activity of 4.7 mCi/mM was added to the above solution. Five ml aliquots of the cell suspension were removed at hourly intervals, and centrifuged at 1,000 $\times g$ for 10 min. The pellet was washed twice in cold distilled water with shaking, and centrifuged. The resultant pellet was suspended in cold 5% trichloroacetic acid (TCA) for 2 hr, and centrifuged. Two additional TCA extractions were made. The extracted pellet was placed in cold redistilled methanol for approximately 30 min, and centrifuged. The radioactivity in the pellet was determined as described in the methods for determining ¹⁴C-glucose leakage. As a reference, the radioactivity in the supernatant was monitored at each step of the above experiments.

Uridine incorporation. Mycelial cells of *F. oxysporum* f. *cucumerinum* (10 g, fresh weight) were incubated in 100 ml of phosphate buffer solution (pH 6.5) containing 0.25 m μ M 5-³H-uridine (specific activity of 18.7 Ci/mM) and or 100, 300 or 1,000 μ g/ml hymexazol. Five ml aliquots of the cell suspension removed at hourly intervals were centrifuged at 1,000 $\times g$ for 10 min. The pellet was rinsed in the distilled water, and centrifuged. The pellet was extracted with 5% TCA,

and centrifuged. The radioactivity of 1/5 of the extracted cells was obtained by the method mentioned above, by which providing levels incorporated into the RNA and the DNA fraction. Radioactivity incorporated into the DNA fraction was determined as follows; a 4/5 of the extracted cells was incubated overnight at 37 C in 0.5 N NaOH. The solutions were neutralized with HCl, and cold TCA (5% final concentration) was added to precipitate the DNA. The samples were centrifuged, and the precipitated DNA and mycelial cells were rinsed with methanol and suspended in scintillation fluid. The amount of radioactivity in the RNA fraction was calculated by subtracting the counts in the DNA fraction from those in the RNA plus DNA fractions.

Thymidine incorporation. Mycelial cells of *F. oxysporum* f. *cucumerinum* (10 g, fresh weight) were incubated for 2 hr in 100 ml of phosphate buffer solution (pH 6.5) containing 0.04 μ M $2\text{-}^{14}\text{C}$ -thymidine (specific activity 37 mCi/mM), and then 100, 300 or 1,000 μ g/ml of hymexazol was added. Five ml aliquots of the cell suspension removed at hourly intervals were washed in the distilled water and centrifuged. The pellet was suspended in 0.5 N NaOH, and incubated overnight at 37 C. The solutions were neutralized, and then the samples were processed and counted according to the procedure described above for determining incorporation of radioactivity into the DNA fraction.

Experiments on phenylalanine, uridine and thymidine incorporations were performed in triplicate, and these experiments were also carried out with *Pellicularia filamentosa* (Pat.) Rogers f. *sasakii* (Shirai) Exmer⁶⁾.

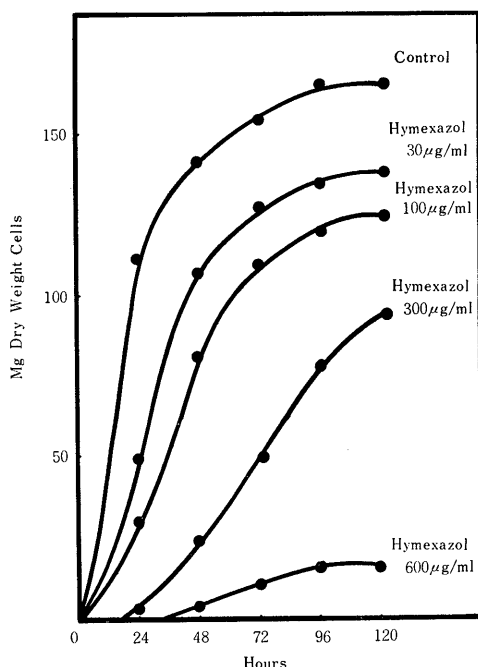


Fig. 1. Growth inhibition of *F. oxysporum* f. *cucumerinum* by hymexazol.

Hymexazol was incorporated into 10 ml of liquid medium in a L-flask. Following inoculation, the flask was incubated for 1-5 day at 27 C on reciprocal shaker operating at 140 rpm.

Results

Effect of hymexazol on the growth of *F. oxysporum* f. *cucumerinum*

The growth of *F. oxysporum* f. *cucumerinum* from mycelial cells was inhibited approximately 50% by hymexazol at a concentration of 300 μ g/ml (Fig. 1). Effect of hymexazol on the growth of various organisms, such as *Pellicularia sakaii*, *Fusarium* spp., *Rhizoctonia* spp., *Saccharomyces cerevisiae* and *Pseudomonas cruciviae*, has been described previously⁶⁾. Of these microorganisms, *S. cerevisiae* was most insensitive to the toxicant, and could grow even at levels up to 1,000 μ g/ml of hymexazol.

Effect of hymexazol on respiration of *F. oxysporum* f. *cucumerinum*

Oxygen uptake by the starved cells of *F. oxysporum* f. *cucumerinum*, which were suspended in phosphate buffer solution (pH 6.5) minus a carbon and nitrogen source for 48 hr prior to making respiration measurement, was not much affected by hymexazol at

a concentration of 500 $\mu\text{g/ml}$ which almost completely suppressed mycelial growth for 36 hr. Antimycin A (5×10^{-5} M) greatly suppressed the respiration of this fungus (Fig. 2-A).

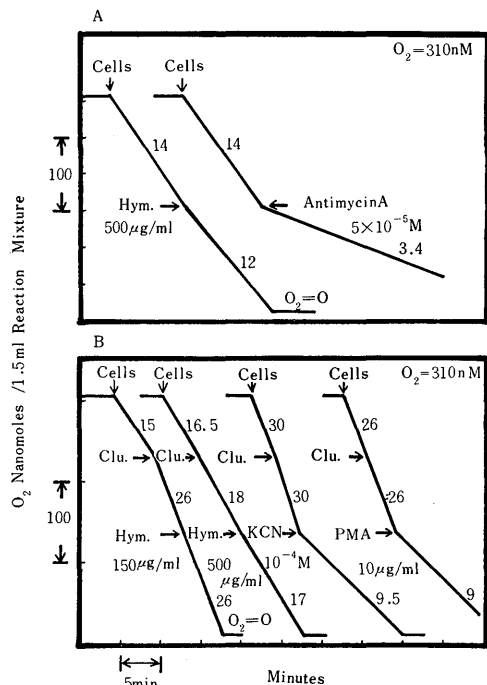


Fig. 2. Effect of hymexazol on respiration of *F. oxysporum* f. *cucumerinum*.

Polarographic traces illustrating the effect of hymexazol on respiration were shown. A: The starved cells were prepared by preincubation for 48 hr in phosphate buffer solution (pH 6.5) without addition of nutrients prior to testing. B: The intact cells were tested in the presence of glucose. Reaction mixture contained 2.0 mg (dry weight) of mycelial cells. Cells, toxicant and substrate were added at the time as indicated by the arrow. Inhibition (oxygen consumption) is shown on the slope in the chart. Numbers along traces are rates expressed as nanomoles $\text{O}_2/\text{min}/1.5$ ml reaction mixture. Glu.; glucose. Hym.; hymexazol. PMA; phenylmercuric acetate. Additions are shown as final concentration.

The inhibitory effect on respiration in the intact cells was monitored upon addition of the toxicant and a time lag was observed with intact cells and mitochondria. Inhibitory effect of hymexazol on the respiration depends on the substrate used. Among the substrates tested, glucose was the most sensitive to the oxidation of intact cells. Succinate was the most sensitive substrate to the oxidation by mitochondria. Oxygen uptake by the intact cells was promoted by addition of glucose. KCN (10^{-4} M) and PMA (phenylmercuric acetate) ($10 \mu\text{g/ml}$) inhibited oxygen uptake by the intact cells, however, hymexazol (150 and $500 \mu\text{g/ml}$) was ineffective (Fig. 2-B).

Effect of hymexazol on the oxidation and phosphorylation in mitochondria

To ascertain the effect of hymexazol on fungal respiration, experiments were performed with the mitochondrial system of the fungus. The oxidative activity of the fungal mitochondria was preliminarily investigated by monitoring the conversion of ADP to ATP in the presence of succinate.

The rates of oxidation of succinate in the hymexazol-treated ($500 \mu\text{g/ml}$) and untreated mitochondria were almost the same. On the other hand, Antimycin A (2×10^{-5} M) completely inhibited the oxidation of succinate in mitochondria (Fig. 3).

Effect of hymexazol on phosphorylation was examined by the successive addition of hexokinase and glucose, ^{32}P , succinate and ADP to the fungal mitochondria in the reaction mixture in the vessel using the methods of Takahashi¹⁷⁾. Hymexazol ($500 \mu\text{g/ml}$) had no effect on oxidative phosphorylation coupled with oxidation of succinate.

Effect of hymexazol on the oxidation in rat mitochondria

For a determination if the effect of hymexazol was inhibitory on respiration, the

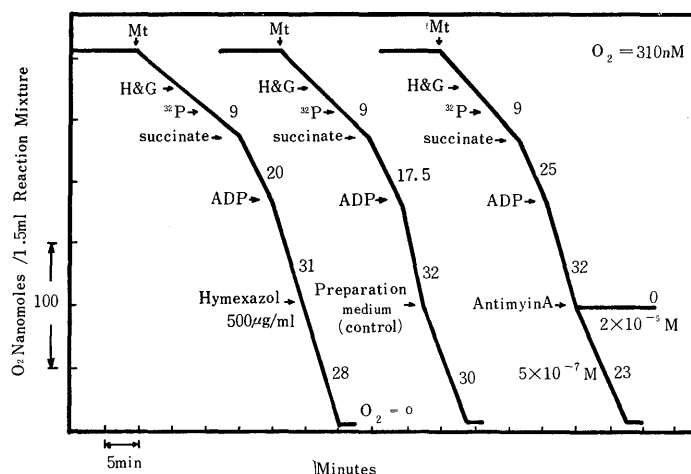


Fig. 3. Effect of hymexazol on oxidation in *F. oxysporum* f. *cucumerinum*-mitochondria. Incubation mixture contained 1.5 mg (dry weight) of mitochondrial protein in a total volume of 1.5 ml. Mt; mitochondria, H & G; hexokinase and glucose (10 μ l), succinate (20 μ l), ADP; adenosine diphosphate (10 μ l), and hymexazol and Antimycin A (15 μ l) were added at the time as indicated by the arrow. Inhibition was shown as in Fig. 2.

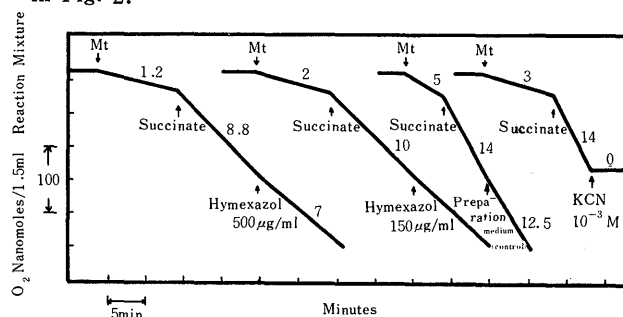


Fig. 4. Effect of hymexazol on oxidation in rat mitochondria. Incubation mixtures contained 5 mg (dry weight) of protein of rat mitochondria, substrate (20 μ l of succinate) and inhibitors (15 μ l). These were added at the time as indicated by the arrow. Inhibition was shown as in Fig. 2.

oxidative activity of rat mitochondria was further investigated under the influence of the toxicant.

Rat mitochondria were also capable of demonstrating ADP response in the presence of succinate. KCN (10^{-3} M) completely inhibited the oxidation of succinate in rat mitochondria, but hymexazol did not (Fig. 4).

Effect of hymexazol on the cell membrane function

The possibility that hymexazol causes damage primarily to the cell membrane was investigated in a manner reported for polymyxins¹²⁾, nystatin¹⁾, and in other antibiotics. The fungal cells had been incubated for 2 hr with 14 C-glucose, and then almost all of the radioactive materials onto the cell surface were removed by repeated washing. The amount of isotope taken up by the cells was 0.07 μ M of 14 C-glucose. Some cells were treated by hymexazol (50 and 500 μ g/ml) or p-s-butylphenol (200 μ g/ml) and the amounts of isotope that leaked out of the cell

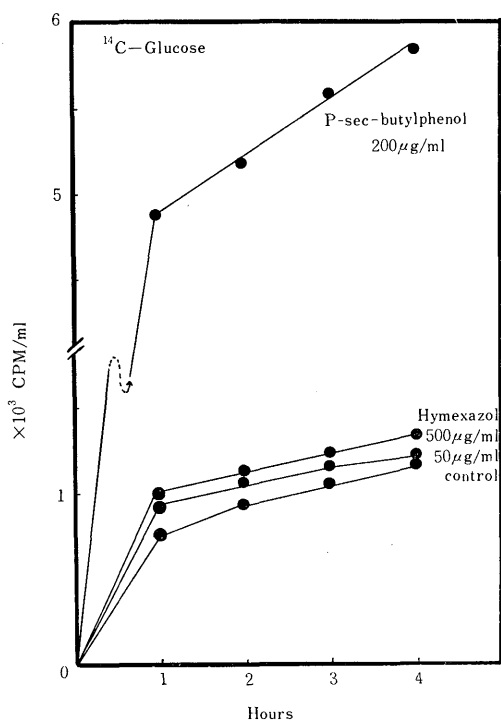


Fig. 5. Effect of hymexazol on the cell membrane function of *F. oxysporum* f. *cucumerinum*.

Fungal cells were incubated for 2 hr at 27 C with ^{14}C -glucose- (U) (specific activity 3.9 mCi/mM; 12 $\mu\text{M/g}$ fresh weight cells) by shaking. The cells filtered off from the medium were washed ten times in buffer, re-suspended in the buffer containing hymexazol (50 and 500 $\mu\text{g/ml}$) or p-s-butylphenol (200 $\mu\text{g/ml}$).

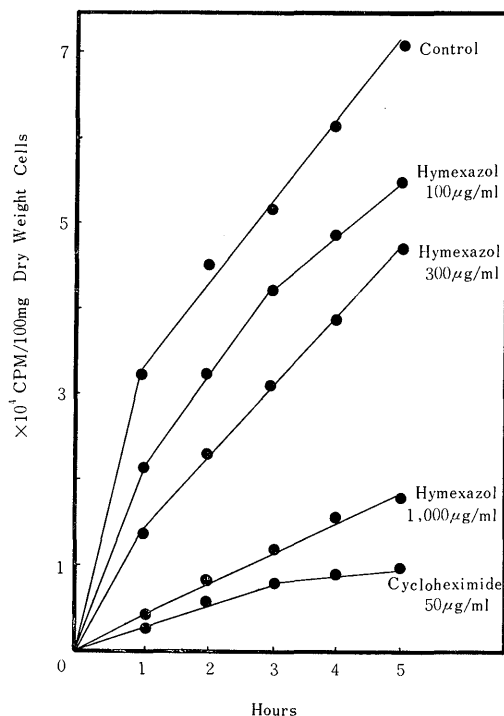


Fig. 6. Effect of hymexazol on phenylalanine incorporation into protein fraction of *F. oxysporum* f. *cucumerinum*. Fungal cells (10 g fresh weight) were incubated for 3 hr at 27 C in 100 ml of phosphate buffer solution (pH 6.5) in a 500 ml-Sakaguchi flask containing 100–1,000 $\mu\text{g/ml}$ hymexazol prior to adding 0.8 μM 3- ^{14}C -L-phenylalanine (specific activity 4.7 mCi/mM).

membranes were measured at hourly intervals (Fig. 5). It was observed that ^{14}C -glucose already taken into the cells did not leak, even at a concentration of 500 μg of hymexazol per ml. A reference compound, p-s-butylphenol, on the other hand, caused great damage to the cell membrane, and resulted in the leakage of ^{14}C -materials out of the cells into the medium (Fig. 5). When ^{32}P was used, the result was quite similar to the above. Thus it is concluded that hymexazol does not cause damage primarily to the cell membranes.

Effect of hymexazol on phenylalanine incorporation

Fungal cells, which had been treated with hymexazol for 3 hr, were incubated with 0.8 μM 3- ^{14}C -phenylalanine. Incorporation of L-phenylalanine into the TCA and methanol insoluble fraction of mycelial cells of *F. oxysporum* f. *cucumerinum* did not show significant inhibition (20%) with 300 $\mu\text{g/ml}$ hymexazol. Inhibition (nearly 70%) was observed with 1,000 $\mu\text{g/ml}$ hymexazol (Fig. 6).

Effect of hymexazol on uridine incorporation

Hymexazol was added to the cells in the medium containing 0.25 m μM 5- ^3H -

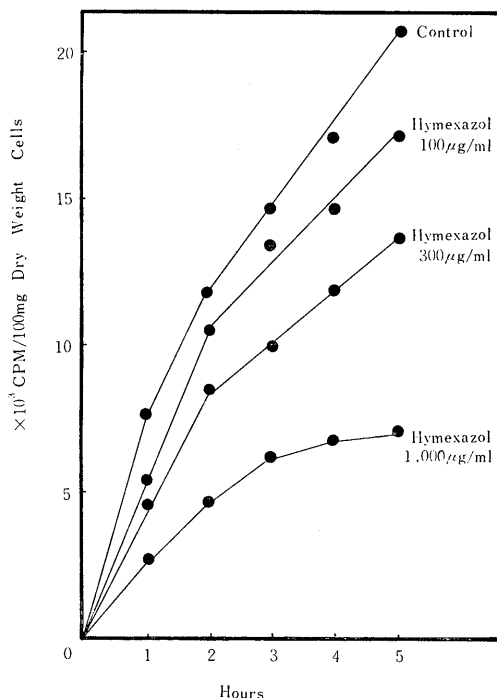


Fig. 7. Effect of hymexazol on uridine incorporation into RNA fraction of *F. oxysporum* f. *cucumerinum*. Fungal cells (10 g fresh weight) were incubated in the medium containing 0.25 μM $5\text{-}^3\text{H}$ -uridine (specific activity 18.7 Ci/mM) and or hymexazol (100–1,000 $\mu\text{g}/\text{ml}$).

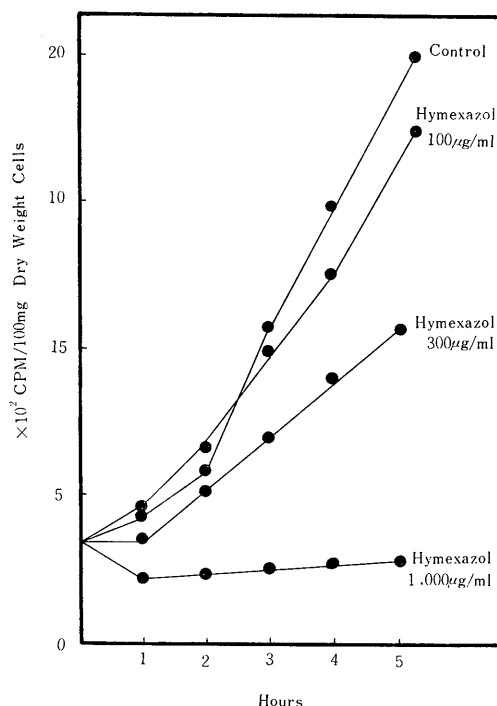


Fig. 8. Effect of hymexazol on thymidine incorporation into DNA fraction of *F. oxysporum* f. *cucumerinum*. Fungal cells (10 g fresh weight) were incubated for 2 hr with 0.04 μM $2\text{-}^{14}\text{C}$ -thymidine (specific activity 37 mCi/mM) prior to adding hymexazol (100–1,000 $\mu\text{g}/\text{ml}$).

uridine, and counts incorporated into the fungal RNA fraction were measured periodically (Fig. 7). Incorporation of uridine into the RNA fraction of mycelial cells of *F. oxysporum* f. *cucumerinum* was inhibited nearly 30% by 300 $\mu\text{g}/\text{ml}$ hymexazol during the period of the experiments, 70%, by 1,000 $\mu\text{g}/\text{ml}$.

Effect of hymexazol on thymidine incorporation

Hymexazol was added to the medium, in which the fungal cells were being incubated with 0.04 μM $2\text{-}^{14}\text{C}$ -thymidine, and the amounts of the isotope in the DNA fraction of the cells were measured periodically (Fig. 8). Incorporation of thymidine into the DNA fraction of mycelial cells of *F. oxysporum* f. *cucumerinum* was considerably inhibited (50%) by 300 $\mu\text{g}/\text{ml}$ hymexazol, and at a concentration of 1,000 $\mu\text{g}/\text{ml}$ no thymidine incorporation was observed (Fig. 8).

Discussion

Hymexazol seemed not to be a strong fungicidal toxicant *in vitro*, but was fungistatic⁶⁾. However, it at 300 $\mu\text{g}/\text{ml}$ considerably suppressed the mycelial growth of *F. oxysporum* f. *cucumerinum* in liquid culture, whereas *S. cerevisiae* was essentially unaffected by concentrations of the toxicant as high as 1,000 $\mu\text{g}/\text{ml}$. The cells of *S. cerevisiae* could grow at more than 1,000 $\mu\text{g}/\text{ml}$ of hymexazol, and even

at 3,000 $\mu\text{g/ml}$ of the toxicant, *S. cerevisiae* could survive with difficulty.

Absorption of hymexazol from the medium by *F. oxysporum* f. *cucumerinum* was significant, whereas *S. cerevisiae* absorbed a little the toxicant⁶⁾. Rate of uptake of hymexazol seemed to be the primary factor determining sensitivity to the toxicant. The lack of ability to metabolize hymexazol does not seem to be the primary factor stated above (Kamimura *et al.*, unpublished data). This was also the case of chloroneb reported by Hock and Sisler³⁾.

Hymexazol did not significantly inhibit respiration of the intact cells as oxidation of succinate in the mitochondria of *F. oxysporum* f. *cucumerinum*, as well as in rat mitochondria proceeded at 150 and 500 $\mu\text{g/ml}$ of the toxicant.

The function of cell membrane of this fungus was not damaged by the concentrations of hymexazol as high as 500 $\mu\text{g/ml}$. This was further studied with more sensitive fungus, *Pellicularia sasakii*⁶⁾, than *F. oxysporum* f. *cucumerinum* by the same experimental system. *P. sasakii* showed the leakage through cell membrane at 500 $\mu\text{g/ml}$ of hymexazol, but, not at 50 $\mu\text{g/ml}$. However, when compared with the effect of p-s-butylphenol (100 $\mu\text{g/ml}$), the effect of hymexazol (500 $\mu\text{g/ml}$) on the cell membrane was still very low.

Interesting evidence for the selective toxicity to fungi of hymexazol was observed in the rate of incorporation of hymexazol in the TCA and methanol insoluble fraction of the cells of the fungi. Sensitive organisms (*P. sasakii* and *F. oxysporum* f. *cucumerinum*) incorporated ¹⁴C-hymexazol into the TCA and methanol insoluble fraction more than insensitive organism (*S. cerevisiae*) did⁶⁾.

With regard to the effect of hymexazol on several metabolic processes in *F. oxysporum* f. *cucumerinum*, the incorporation of thymidine into DNA was inhibited to a degree that might be expected to lead to a suppression of growth. And the incorporation of phenylalanine into protein and uridine into RNA were also inhibited considerably, but, lesser extent than that of thymidine into DNA. This was the same with *P. sasakii*, when tests were made with the same system in F-medium, instead of the buffer solution.

The findings in this report suggest that hymexazol may exert its antifungal activity by interfering with DNA metabolism.

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和文摘要

土壤殺菌剤，ハイメキサゾール，3-ヒドロキシ-5-メチル イソキサゾールのキウリ蔓割病菌に対する殺菌作用機序

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土壤殺菌剤，ハイメキサゾール (3-hydroxy-5-methylisoxazol) の殺菌作用機序について，感受性菌 *Fusarium oxysporum* f. *cucumerinum* を用いて検討した。非感受性菌 *Saccharomyces cerevisiae* を対照菌として用いた。前者は液体培養条件下で，ハイメキサゾールの濃度 300 μ g/ml で生育がかなり抑制されたが，後者は 1000 μ g/ml の濃度においてもなお生育した。これら菌種間でのハイメキサゾールの選択毒性の差は，主に菌種間での本薬剤の吸収量の差異に起因している。*S. cerevisiae* は，本薬剤添加培養 (5hr) において，特に薬剤代謝転換を示さなかった。

ハイメキサゾールの *F. oxysporum* f. *cucumerinum* に対する殺菌作用機作を検討した結果，本菌の生菌に対する呼吸阻害は少なく，また本菌のミトコンドリアの酸化的リン酸化反応の阻害も少なかった。さらに，ラットのミトコンドリアの酸素吸収に対する阻害作用も，ほとんどみられなかった。ハイメキサゾールが，本菌に対して強い生育阻害を示す濃度，500 μ g/ml において，本菌々体膜の透過性の阻害を検討したが，対照薬剤，p-s-butylphenol に比較すると，本薬剤の阻害作用は少ないものであった。本菌々体蛋白分画へのフェニールアラニンの取りこみ，及び RNA 分画へのウリデンの取りこみを検討したところ，ハイメキサゾールによりかなりの阻害がみられた。また本菌々体の DNA 分画へのチミデンの取りこみ阻害を検討したところ，フェニールアラニン及びウリデンの菌体蛋白分画及び RNA 分画への取りこみより，より強い阻害を本薬剤は示した。これらの結果より，ハイメキサゾールの殺菌作用機序として，*F. oxysporum* f. *cucumerinum* の核酸代謝，また蛋白代謝の阻害が考えられる。